

Assay of Cyclic AMP in Lysates of Cells
AfCS Procedure Protocol ID PP0000001500
Version 1, 03/07/02

The method chosen for measuring the content of cyclic adenosine 3',5'-monophosphate (cyclic AMP or cAMP) in splenic B lymphocytes (B cells) is an enzyme-linked immunoassay developed by Amersham Biosciences. An overview of the assay, [cAMP Biotrak EIA](#), and its instructions, [Biotrak Protocol](#), are available through the indicated links. The procedure described below follows the basic instructions of the Biotrak System, Protocol 2, for the analysis of total cAMP in cultured cells (includes extracellular and intracellular cAMP). This protocol utilizes acetylation of cAMP to improve sensitivity and reduce interference. Lysates of cells are made and stored at -80 °C (see AfCS Protocol *Preparation of B-Lymphocyte Lysates for Cyclic AMP Determination*, PS0000001200).

Determination of cAMP

1. Prepare all buffers and reagents required for the enzyme-linked immunoassay (EIA) of acetylated cAMP as described in Protocol 2 in the [Biotrak Protocol](#). Reagents provided in the kit are listed in the *Reagents and Materials* section below.
2. Equilibrate all reagents at room temperature and mix thoroughly before use.
3. Prepare standards of cAMP by reconstituting the stock acetylation standard with assay buffer as directed, and dilute this stock solution (2.56 pmol cAMP/ml) to concentrations of 0.64, 0.32, 0.16, 0.08, 0.04, and 0.02 pmol/ml by serial dilution of 400 µl into 400 µl of assay buffer. Use assay buffer for the 0 cAMP standard.
4. Plan the 96-well microtiter-coated plate to contain duplicate wells for blanks, each standard, and nonspecific binding (NSB) controls (these include cAMP-peroxidase but not antibody). Unknown samples can be measured as desired.
5. Resuspend dried samples with 100 µl of assay buffer and mix thoroughly. Remove any particulate debris by centrifugation for 2 min at 14,000 rpm in a microfuge, and keep on ice or at 4 °C until ready to use.
6. Dilute the unknown samples with assay buffer in appropriately labeled fresh Eppendorf tubes to produce final volumes of 400 µl. (A 1:10 dilution is commonly used for samples that contain basal concentrations of cAMP, while greater dilutions are used for samples in which cAMP is elevated).
7. Prepare the acetylation reagent by mixing 1 volume of acetic anhydride with 2 volumes of triethylamine in a glass tube.
8. Carefully add 10 µl of acetylation reagent to all tubes including standards and unknown samples. Vortex **each tube** immediately following the addition of the acetylation reagents.
9. Pipette 100 µl of antiserum into all wells to be used except the blank and NSB wells.
10. Pipette 50 µl of acetylated standards and unknown samples into the appropriate wells.

11. Pipette 150 μ l of assay buffer into the NSB wells.
12. Cover the plate with the lid and shake at 400 rpm on a titer plate shaker for 5 sec. Incubate at 4 °C for exactly 1 hr.
13. Dilute the stock of cAMP-peroxidase conjugate with an equal volume of assay buffer.
14. Pipette 100 μ l of the diluted cAMP-peroxidase conjugate into all wells except the blanks.
15. Cover the plate with the lid, mix as above and incubate at 4 °C for 1 hr.
16. Remove the reaction mixture by flicking the plate in the sink and blot the plate vigorously on dry paper towels. Wash all wells four times with 400 μ l aliquots of wash buffer. (Note: the plate contains removable strips of wells that should be labeled, as the strips can detach if blotting vigorously.)
17. Immediately dispense 150 μ l of enzyme substrate into all wells in a timed sequence with a multichannel pipette.
18. Cover the plate and shake at 400 rpm on a titer plate shaker for 30 min at room temperature.
19. Pipette 100 μ l of 1 M sulfuric acid into each well with a multichannel pipette in the same timed sequence as for the addition of substrate (step 17). Mix by shaking at 400 rpm on a titer plate shaker for 10 sec. Read the absorbance of each well at 450 nm within 30 min.

Calculations

20. A standard curve for cAMP is generated by plotting the % B/Bo versus the cAMP standard (plotted as fmol; 0-128), where B = sample OD at 450 nm and Bo = OD of cAMP standard at 0 pmol/ml. The data are fit with a logistic 4-parameter regression analysis.
21. The cAMP (fmol) in unknown samples is calculated from the standard curve and the size of the aliquot measured.
22. The cAMP concentrations are expressed as either "pmol/million cells" or "fmol/ μ g protein." Calculations are based on the number of cells dispensed into the wells or a direct determination of protein content in the sample by the Amido black protein assay (see AfCS Protocol *Amido Black Assay*, PP0000002100).

Reagents and Materials

cAMP Biotrak EIA assay kit: Amersham Biosciences; catalog no. RPN225
Includes assay buffer; lysis reagents; acetylation reagents; cAMP standards; antiserum; cAMP-peroxidase conjugate; wash buffer; 96-well microtiter-coated plate; and enzyme substrate; prepared as directed in the instruction booklet [Biotrak Protocol](#).

Sulfuric acid, 1 M: AfCS Solution Protocol ID PS0000003100

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